A STABLE POLLEN ANTIGEN *

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The early work on the preparation of pollen extracts[1] brought to light the fact that aqueous extracts are unstable and rapidly deteriorate, losing their potency after about 3 weeks. This point is mentioned by Dunbar,[2] and especially emphasized by Koessler,[3] who states:

> The pollen extract is not stable, especially not the higher dilutions. By progressing proteolysis, after 3 to 4 weeks, it acquires marked toxic properties which lead to severe reactions. The solution must, therefore, be freshly prepared every 8 to 10 days if these reactions are to be avoided. . . . The material to be injected must be not only sterile, but constantly of uniform potency if used in the same dilution. No extract of pollen can comply with this demand if it is older than three weeks.

It appeared, therefore, that extracts of pollen could not be widely used by physicians unless some method of extraction could be devised which would insure a uniformly stable product. With this end in view, we have prepared pollen antigens which we have subjected to rigid tests for stability.

Pollen from the flowering plants was obtained in the same manner, beginning May 23, 1914, and continuing until Sept. 9, 1914. The plants were closely observed and, as soon as some of the pollen pods began to open, the flowering tops were cut off with suitable stems so that the cut flowers could be easily placed with the cut end of the stem in water, the flower hanging over the edge of the basin. The basins were set in well-lighted rooms, carefully protected from currents of air, on long tables covered with clean smooth paper. As the flowering tops, which continued to 'live' for a short period, began to drop pollen on the paper, the pollen was carefully collected with camel's hair brushes and dried in thin layers in vacuum dessicators over sulphuric acid, at room temperature, in the dark. The pollen was allowed to remain in the dessicators for a month or more, when it was collected in bulk in glass jars, which were stored in dessicators. In this dry state the pollen does not deteriorate: according to Goodale,[4] it has been kept 25-30 years without any loss in antigenic power. All antigens have been freshly prepared from this stock of dried pollen.

* Received for publication June 9, 1917.

[1] Lancet, 1911, p. 1572. Ibid., 1911, 2, p. 814. Proc. Soc. Exper. Biol. and Med., 1913, 10, p. 70.

[2] Jour. Hyg., 1913, 13, p. 105.

[3] Forchheimer's Therapeusis of Internal Diseases, 1914, 5, p. 671.

[4] Boston Med. and Surg. Jour., 1915, 173, p. 42.

A mixture was made of equal parts by weight of the pollens of timothy, red top, June grass, orchard grass, rye, sorrel dock, daisy, maize, ragweed, and goldenrod; these being the pollens which are known to cause the greatest number of cases of the vernal and the autumnal type of hay-fever.

Since, in the experimental work reported by others[5] in this country, the pollen was extracted in salt solution, we first prepared antigens after this method in order to test for stability.

Antigen 10.—On Jan. 29, 1915, 2.03 gm. of the pollen mixture mentioned were ground with glass dust in a mortar, using 9% salt solution to moisten the mixture which was ground for 2½ hours. A sufficient amount of 9% salt solution was added to bring the total volume up to 145 c.c., which according to Koessler, Noon, Freeman, and others gives a dilution so that 1 c.c. is equivalent to approximately 14,000 units of pollen: the pollen unit having been arbitrarily chosen by those workers as the equivalent of one-millionth gm. of pollen. The mixture was shaken for 30 minutes and placed at 37 C. for 16 hours. It was then shaken for 1 hour, centrifugalized, and passed through Buchner and Berkefeld filters. Trikresol was added to 0.3% as a preservative.

After being prepared, the antigen was kept in an ice-box at about 5 C. Before being titrated, a 10% dilution of the antigen was made using sterile distilled water as diluent, thus making the antigen isotonic, and then further diluted to 1% with physiologic salt solution.

Using the same technic for complement fixation titrations as that adopted by the Research Laboratories of the Department of Health, New York City, 1 pollen unit was found to be equivalent approximately to $\frac{1}{25}$ of a unit of Antigen 10 (Table 1), taking a unit of antigen as the smallest amount that gives complete fixation in the hemolytic series. Immune serum was obtained from rabbits which had been immunized with a gradually increasing number of units of pollen. Table 1 shows that 0.000025 gm. of pollen is the antigen unit (smallest amount that gives complete fixation). Since the pollen unit is taken as 0.000001 gm. of pollen, it follows from this table that 1 pollen unit is equivalent to $\frac{1}{25}$ of a unit of antigen. With this as a basis for measuring the strength of the antigen, pollen extracted in 9% salt solution gave an antigen that deteriorated quite rapidly. Within 16 days, 40% disintegrated; at the end of 1 month, about 50%; while at the end of 7 weeks, only traces of antigenic properties remained (Table 2).

Eleven antigens were prepared and tested as outlined, but all showed the same rapid deterioration. This confirmed Koessler's statement that the saline extracts are unstable.

[5]Forchheimer's Therapeusis of Internal Diseases, 1914, 5, p. 671. Jour. Am. Med. Assn., 1914, 63, p. 141.

Inasmuch as glycerol has been successfully used for many years to preserve the standard diphtheria antitoxin, it was decided to incorporate glycerol in the method of preparing pollen antigens in order to prevent, if possible, the rapid deterioration of pollen protein. Accordingly, the next antigen was prepared by extracting in salt and glycerol as follows:

Antigen 12.—On Feb. 26, 1915, 2.98 gm. of the dried pollen mixture already described were extracted with 213 c.c. of a diluent composed of 33⅓% saturated sodium chlorid solution and 66⅔% glycerol. Essentially the same technic was

TABLE 1

TITRATION OF ANTIGEN 10 (SALINE EXTRACT) FEBRUARY 3, 1915

Number of Tube	Immune Serum, C.c.	Antigen		10% Complement, C.c.	0.9% Salt Solution, C.c.	Sensitized Erythrocyte Suspension, C.c.	Results
		1:100 C c.	Grams of Pollen				
1	0.01	0.25	0.000025	0.1	0	0.2	++++
2	0.01	0.20	0.000020	0.1	0	0.2	+++
3	0.01	0.15	0.000015	0.1	0.05	0.2	++
4	0.01	0.10	0.000010	0.1	0.10	0.2	—
5	0.01	0.05	0.000005	0.1	0.15	0.2	—
6	0.01	0.025	0.0000025	0.1	0.20	0.2	—
7	0	0.4		0.1	0	0.2	—
8	0	0.3		0.1	0	0.2	—
9	0	0.2		0.1	0.05	0.2	—
10	0	0.1		0.1	0.10	0.2	—
11	0	0.05		0.1	0.15	0.2	—
12	0.02	0		0.1	0.20	0.2	—

In these tables, Citron's standard for the strength of a reaction is used: namely, complete absence of hemolysis is indicated by a 4-plus sign (++++); faint hemolysis is shown by a 3-plus sign (+++); partial hemolysis is shown by a 2-plus sign (++); nearly complete hemolysis is indicated by a single plus sign (+); doubtful binding of complement or practically complete hemolysis is shown by a plus and minus sign (±); while a minus sign (—) indicates complete hemolysis.

The mixtures of immune serum, antigen, and complement were put in the ice-box for 15 hours before adding the sensitized erythrocytes. The results were read after 1 hour at 37 C.

TABLE 2

RESULTS OF FURTHER TITRATION OF ANTIGEN 10

Date of Titration	Antigen		Results
	1-10 C.c.	Grams of Pollen	
Feb. 19, 1915	0.25	0.000025	++
	0.20	0.00002	+
	0.15	0.000015	—
March 1, 1915	0.25	0.000025	+
	0.20	0.000020	—
March 24, 1915	0.25	0.000025	±
	0.20	0.000020	—

followed as described for Antigen 10, except that no preservative was added after the antigen was passed through a Berkefeld filter.

The antigen was stored in the ice-box at 5 C., and before being titrated was rendered isotonic by being diluted to 10% with sterile distilled water, and a further dilution to 1% was made with salt solution.

Complement-fixation titrations, using the technic described, showed that 1 pollen unit was equivalent approximately to $\frac{1}{20}$ of a unit of Antigen 12 (Table 3). With this as a basis for measuring the strength of the antigen, pollen extracted in 66⅔% glycerol and 33⅓% saturated sodium chlorid solution gave an antigen that has proved to be remarkably stable and potent and which remains sterile.

Table 3 shows that 0.000020 gm. of pollen is the antigen unit (smallest amount that gives complete fixation). Since 0.000001 gm. is taken as the pollen unit, it is evident that 1 pollen unit is equivalent to $\frac{1}{20}$ of a unit of Antigen 12.

TABLE 3

TITRATION OF ANTIGEN 12 (GLYCEROL-SALINE EXTRACT) MARCH 1, 1915

Antigen		Results
1:100 C.c.	Grams of Pollen	
0.25	0.000025	++++
0.20	0.000020	++++
0.15	0.000015	+++
0.10	0.000010	++
0.05	0.000005	—
0.025	0.0000025	—

By comparing this table with Table 1, it will be seen that the saline extract (Antigen 10) gave complete fixation in the 1st tube only ($\frac{1}{25}$ of a unit of the antigen equals 1 pollen unit); while the glycerol-saline extract (Antigen 12) gave complete fixation in the 2nd tube also ($\frac{1}{20}$ of a unit of antigen equals 1 pollen unit). Therefore, the glycerol-saline extract is thus shown to be 25% stronger in antigenic power than the saline extract.

Antigen 12 was stored at ice-box temperature, and was subsequently titrated on the following dates:

March 24, 1915
May 6, 1915
May 19, 1915
June 23, 1915
July 19, 1915
Sept. 13, 1915
May 1, 1916

The results of these titrations were exactly like those shown in Table 3. In other words, the antigen remained for 14 months without any deterioration which could be determined by the complement-fixation

test. During that period, anticomplementary properties did not develop. Repeated bacteriologic examinations showed the antigen to be sterile at all times.

From time to time, many other antigens were prepared and tested in the same manner, and all of them exhibited the same uniform stability.

SUMMARY

Pollen antigens, prepared by extracting the dried pollen in 66⅔% glycerol and 33⅓% saturated sodium chlorid solution as herein described, and stored in the ice-box at 5 C., remained without any detectable loss in antigenic properties for 14 months.

Such pollen antigens not only remained remarkably stable, but also were protected from bacterial growth, and did not develop anticomplementary properties.

Pollen extracted in glycerol and salt, as herein described, produced antigens that were 25% stronger in antigenic properties than antigens prepared by extracting the pollen in salt solution only.

Pollen extracted in salt solution gave antigens that deteriorated quite rapidly, only traces of antigenic properties remaining at the end of 7 weeks.